TITLE

2015 Phase 1 EPA/PMRA Summary Report

TEST GUIDELINE

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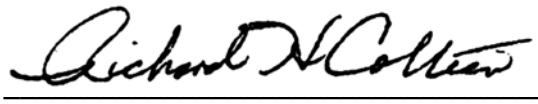

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2015 Phase 1 EPA/PMRA Summary Report:

Proposed Adaptations to the OECD Draft Guidance Document-
"Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure".

Introduction

A collaborative, multi-lab effort was initiated in July 2015 with the goal of implementing method modifications to the "OECD Draft Guidance Document for Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure" (OECD, 2015) developed at the University of Florida (UF) and evaluating control survival in honey bees reared using the UF method modifications. The Phase 1 testing participants included four United States laboratories (California Agricultural Research, Eurofins Agroscience Services, Smithers Viscient, and Syntech) and six European laboratories (BASF SE, Bayer CropScience, BioChem, Eurofins Agroscience Services, Ibacon, and Innovative Environmental Services) with some of the laboratories conducting multiple trials. Each of the European laboratories conducted the UF method modifications in parallel with the current OECD draft guidance procedure to provide a direct comparison between the two methods (Phase 1 methodology described within Appendix A). Additionally, all European laboratories participating in Phase 1 also participated in the initial OECD Draft Guidance Document ring test. Three primary differences between the two methods are as follows:

- **Diet Composition**- UF methodology contains more water and less royal jelly than OECD draft guidance procedure to facilitate diet consumption and limit diet dehydration during exposure
- **Pre-Pupal Transfer**- Transfer of the late stage larvae to a new plate for pupation and adult emergence
- **Rearing Environment**- Lid placed upon larval and pupal plate while in incubator; no glycerol solution used

Results

All data from the participating laboratories were reported regardless of adverse outcomes, including, but not limited to, environmental conditions, equipment failure, or experimental error. Only two of the participating laboratories had experience with the UF method modifications prior to Phase 1 testing. The average years of grafting experience among the European testing laboratories was 3.3 years, whereas the grafting experience within North American testing laboratories was 1.4 years. There were variable rates of success between the testing laboratories, with higher survival rates being reported in Europe relative to the US.

Europe

- Average larval survival (total) on D6-D8 (grafting occurred on D0):
    - UF method modifications: 95.0%
    - OECD: 96.6%

- Average adult emergence (total) recorded on D18-D21:
    - UF method modifications: 83.1%
    - OECD: 85.3%

North America

- Average larval survival (total) on D6-D8 (grafting occurred on D0):
    - UF method modifications: 84.9%
- Average adult emergence (total) recorded on D18-D21:
    - UF method modifications: 59.2%

Chronic larval test performance data were characterized further by calculating pre-pupal survival (6-8 days post grafting) and adult emergence (18-21 days post grafting) across each individual laboratory. In all European labs, the OECD and UF methodology were conducted in parallel to provide a basis of comparison for test performance for pre-pupal survival (Fig. 1) and adult emergence (Fig.2). There is no evidence of significant difference in test performance between the methodologies. North American laboratories tested only the UF methodology (Fig. 3). There were substantial differences in performance, resulting in some laboratories exceeding the validity criterion for adult emergence of ≥70, but others experiencing very poor survival throughout the trial.

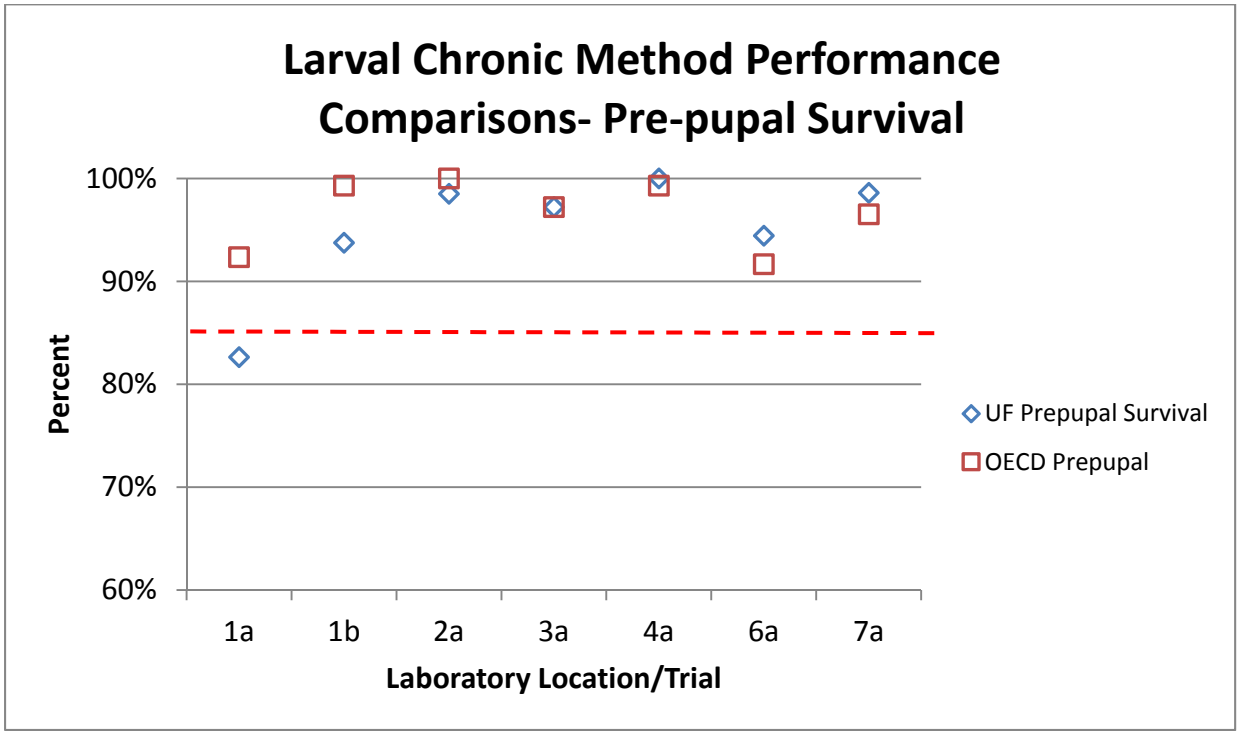

Figure 1. Comparison of chronic larval method pre-pupal survival. OECD (red symbol) and University of Florida (UF, blue symbol) method performance were compared across 6 laboratories (#) and 7 trials (eg. a, b) in Europe. The dashed red line represents the current OECD draft guidance validity criterion for pre-pupal survival of ≥ 85%.

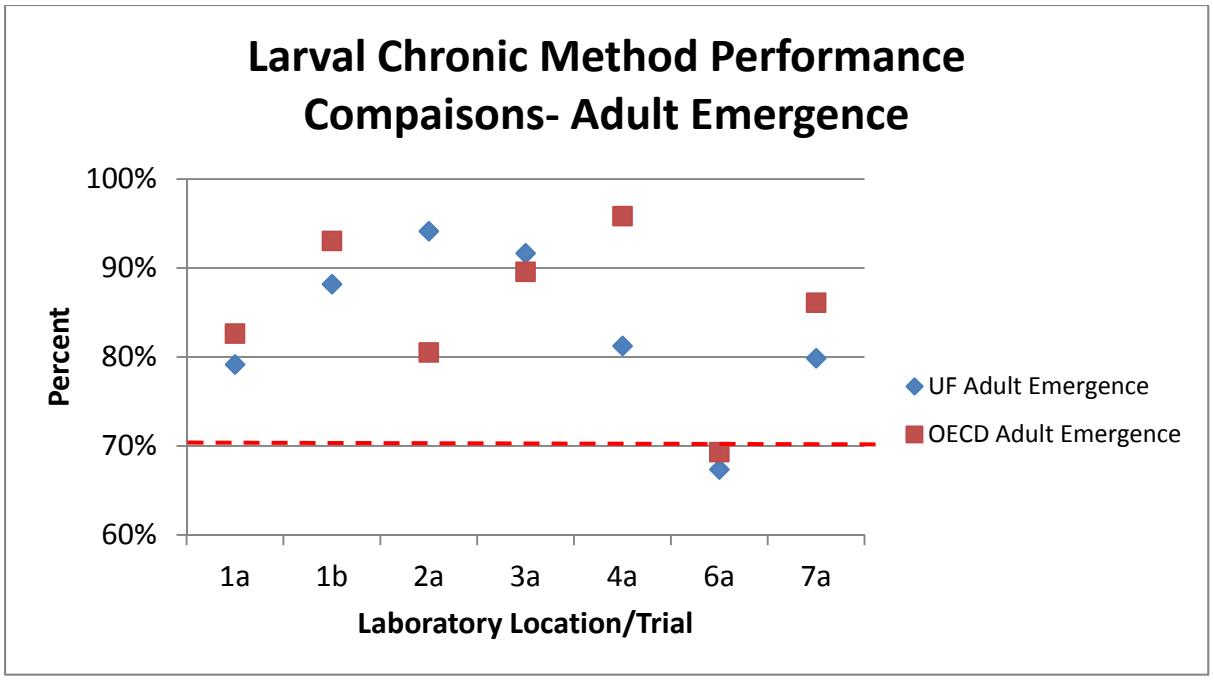

Figure 2. Comparison of chronic larval method adult emergence. OECD (red symbol) and University of Florida (UF, blue symbol) method performance were compared across 6 laboratories (#) and 7 trials (eg. a, b) in Europe. The dashed red line represents the current OECD draft guidance validity criterion for adult emergence of ≥ 70%.

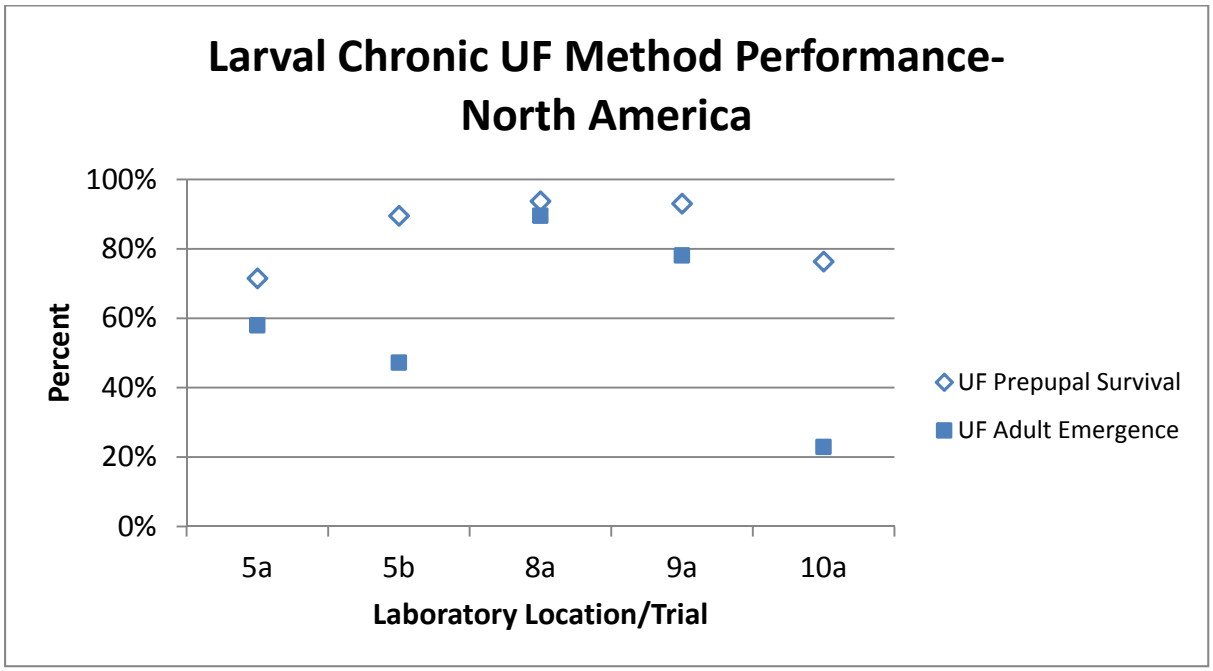

Figure 3. The University of Florida method performance in North America. Pre-pupal (open diamonds) and adult emergence (filled diamonds) performance was characterized across 4 laboratories (#) and 5 trials (eg. a,b).

All participating Phase 1 laboratories were asked to complete a questionnaire with the aim of characterizing the observed differences in test performance. It is unclear what technical and environmental conditions are accounting for differences. Experience was thought to be an important contributor to performance, however a linear regression analysis conducted to characterize the contribution of experience with adult emergence percentage (Fig. 4) found that test performance was not dependent upon experience ($y = 0.016x + 0.6935$, $R^2 = 0.0473$).

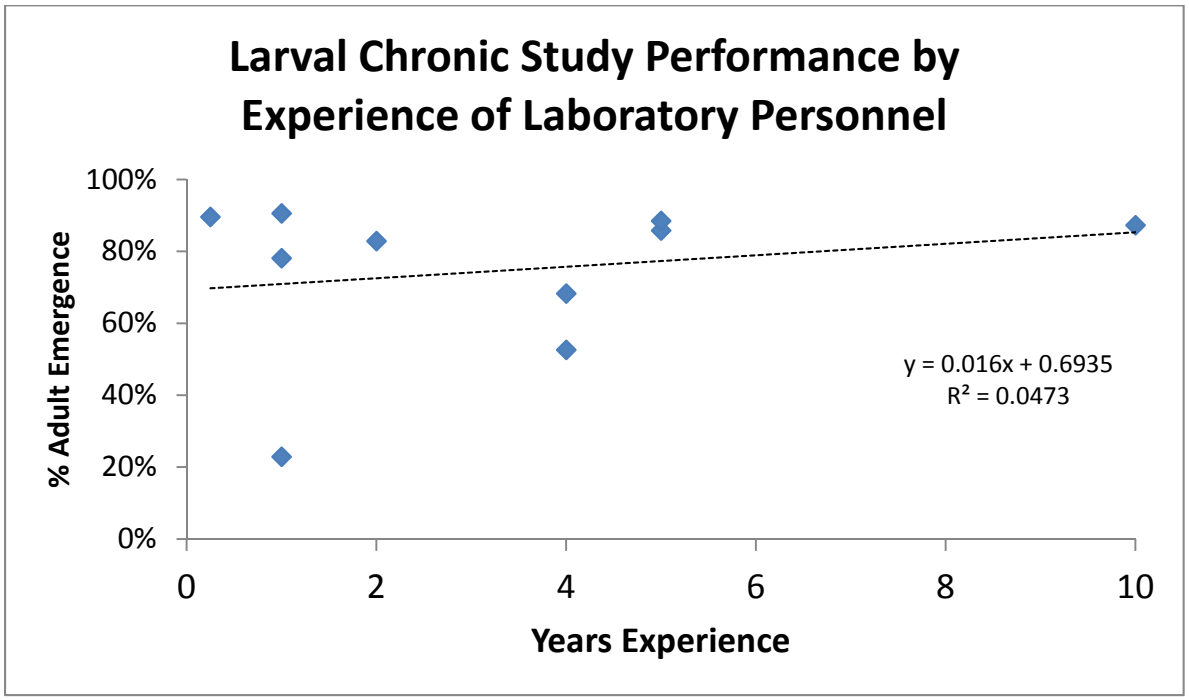

Figure 4. Relationship between laboratory experience and performance. The maximum experience (years) represented within each laboratory was grafted alongside the average percent adult emergence (%), not taking account of which methodology was conducted.

Adult emergence performance data from the Phase 1 testing are summarized (Table 1) with the objective of capturing the Phase 1 variability across trial, replicate (plate), or source colony. As summarized earlier, the two methodologies did not perform differently when conducted in parallel (85.7% of trials were valid for both UF and OECD methodology). Furthermore, the source colony variation did not have any impact on performance (data not shown).

Table 1. Performance of total trials, replicates, and colonies satisfying the validity criterion of ≥ 70% adult emergence. Trials were conducted in Europe (EU) or North America (NA) according to the larval chronic methodology of OECD and/or the University of Florida (UF). Data were summarized A) by trial, B) by replicate (defined by plate), or C) by source colony. Each sub-table is further broken down by region or by method.

A) By trial	Region and/or Method	# Valid	Total Count	% Valid
Calculated by Region	All	14	19	73.7%
	EU	12	14	85.7%
	NA (UF methodology only)	2	5	40.0%
Calculated by Method	UF (EU + NA)	8	12	66.7%
	UF (EU only)	6	7	85.7%
	OECD (EU only)	6	7	85.7%
B) By replicate (defined by plate)	**Region and/or Method**	**# Valid**	**Total Count**	**% Valid**
Calculated by Region	All	40	55	72.7%
	EU	36	42	85.7%
	NA (UF methodology only)	4	13	30.8%
Calculated by Method	UF (EU + NA)	21	34	61.8%
	UF (EU only)	17	21	81.0%
	OECD (EU only)	19	21	90.5%
C) By source colony	**Region and/or Method**	**# Valid**	**Total Count**	**% Valid**
Calculated by Region	All	42	54	77.8%
	EU	38	42	90.5%
	NA (UF methodology only)	4	12	33.3%
Calculated by Method	UF (EU + NA)	23	33	69.7%
	UF (EU only)	19	21	90.5%
	OECD (EU only)	19	21	90.5%

Discussion

While the Phase 1 data do not indicate differences between the two methodologies, feedback was gathered from many of the participating laboratories to provide context and scope to what was observed. The feedback is summarized for each region as follows:

Europe

- OECD draft guidance methodology during Phase 1 near perfection- participants reinforced that fall results were not typical during previous years/initial "ring-test"
    - Previous data from EU in hand demonstrating that only 50% of test runs fulfilled validity criteria
    - First UF methodology trials conducted in EU- feel this constitutes a success despite comparable rates of survival
- Larval diet consumed faster/more completely in UF method than OECD draft guidance
- UF method a bit more effort (transfer step), but testing laboratories are fine with changes and will be willing to adapt tweaks to the current OECD draft guidance
- Difficulty maintaining relative humidity at requested set-points
- Some apparent difficulty in transfer step that may require additional training
- Highest mortality occurring during early stages of pupation across both methods

United States

- Better larval success than any methodology previously utilized
    - High levels of repeatability in definitive tests after implementing UF method modifications
- High levels of variability in results
- Optimism and eagerness to adopt method, but further refinement needed to achieve higher rates of survival and consistency across tests, especially for adult emergence
    - US lagging behind Europe in chronic larval test
- Difficulty maintaining relative humidity at requested set-points

Feedback Summary:

- Although most of the participating labs were conducting the "new method" for the first time, most labs produced a valid (according to recommended validity criteria) test
    - Equal number of valid tests in Europe when the two rearing methods were conducted in parallel
- Method adaptions didn´t lead to much higher emergence rates but they also didn´t lead to lower emergence rates, although a step like larval transfer needs additional training
- **Therefore, it is recommended to conduct a ring test in 2016 to:**
    1. **Prove if good results are repeatable**
    2. **Confirm whether increased experience and training will lead to even higher emergence rates**

Next Steps:

1. Alignment with North American authorities (EPA/PMRA) on chronic larval test design
    - Discussion of variation and difficulties, critical steps and conditions for improving test performance, and feedback from participating laboratories
2. Discussion for alignment and communication strategy between CLA/CLI and OECD authorities
3. Conduct a formal ring-test during 2016 implementing agreed-upon chronic larval methodology
    - Increase number of EU and NA testing laboratories from what was included in initial OECD draft guidance document testing
    - Include all data collected from the ring-test analyses (ie. no data censorship)
    - Include positive control (eg. dimethoate, fenoxycarb, or dimilin) and solvent control
    - Standardize testing conditions (eg. seasonality)

Appendix A. Methods Overview

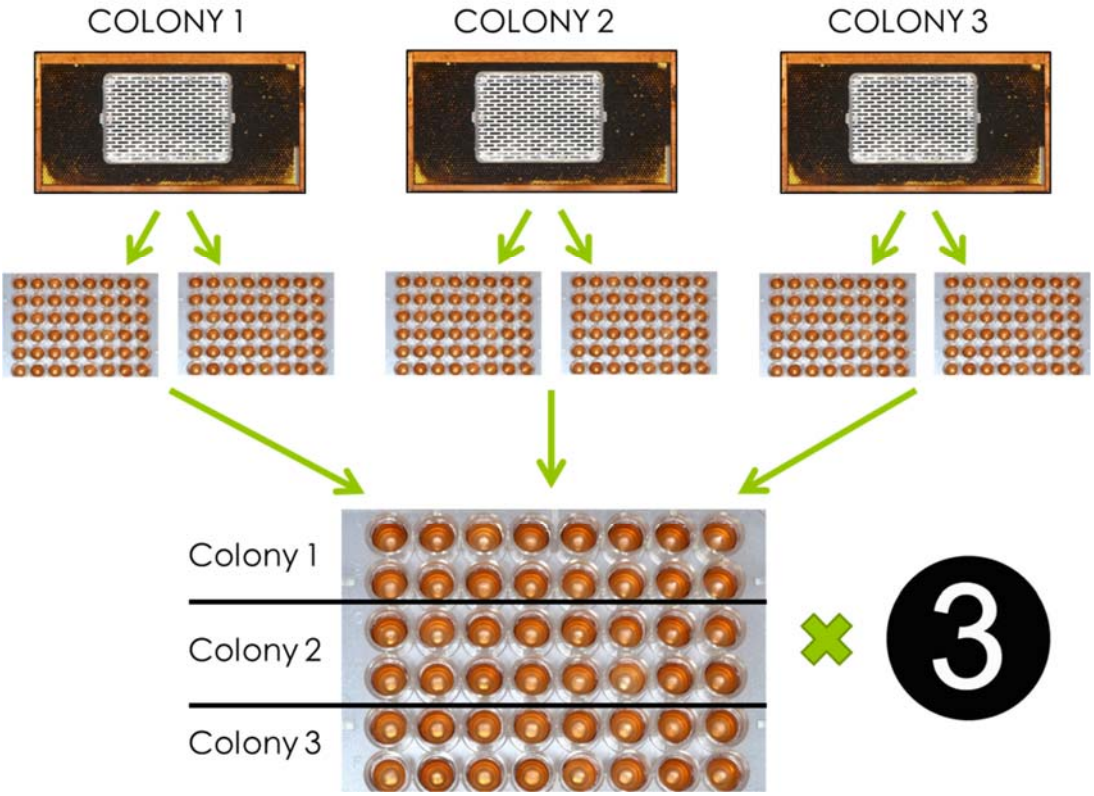

Figure S1. Overview of the Phase 1 experimental design.

Queens will be confined to empty brood comb on one frame per colony in at least 5 colonies for a 24 hour period. After 24 hours, the queen will be released from the queen excluder cage. Once released, the cage will be placed back without the queen to prevent the queen from relaying additional eggs in the same location. After a period of 75 hours (3 days and 3 hours after the queen is released), three frames (one frame/colony for three colonies) with young larvae (1^{st}-2^{nd} instar) are transferred to the laboratory.

Two plates of 48 larvae/plate will be transferred from the frame of each of the three colonies to the larval plates (grafted in excess, totaling 96 larvae/colony for three colonies). On day 2 (D2, two days after grafting), the larvae will be allocated by transferring 16 cell cups with healthy larvae from each colony to a new sterile culture plate so that each plate contains 48 larvae from the three colonies (16 larvae X 3 colonies = 48 larvae). The study will be conducted in triplicate, resulting in a total of three plates with 16 larvae/colony on each plate (Fig S1).

On day 0 (D0) of the study, young larvae (between 1st-2nd instar) of a synchronized age are taken from the comb of three colonies and individually placed into 48-well plates where they are fed a standardized amount of artificial diet through day 5 (D5, five days post grafting). Pre-pupal honey bees (only the bee) are transferred to a new culture plate after their diet is fully consumed (D6-D8, six to eight days post grafting respectively). The bees' development progresses from young larvae (between 1st and 2nd instar) to pre-pupae in the larval plate and continue their pupation in the pupal plate until adult emergence. Larval survival (recorded on D6-D8) and adult emergence (recorded on D18-D21) are reported within the results summary report.